



Disinfection and removal of human pathogenic bacteria in arctic waste stabilization ponds

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2 **Disinfection and removal of human pathogenic bacteria in arctic waste**
3 **stabilization ponds**
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ABSTRACT

Wastewater stabilization ponds (WSPs) are commonly used to treat municipal wastewater in Arctic Canada. The biological treatment in the WSPs is strongly influenced by climatic conditions. Currently, there is limited information about the removal of fecal and pathogenic bacteria during the short cool summer treatment season. With relevance to public health, the objectives of this paper were to determine if treatment in arctic WSPs resulted in the disinfection (i.e., removal of fecal indicator bacteria, *Escherichia coli*) and removal of selected human bacterial pathogens from the treated effluent. The treatment performance, with focus on microbial removal, was assessed for the one-cell WSP in Pond Inlet (NU) and two-cell WSP in Clyde River (NU) over three consecutive (2012-2014) summer treatment seasons (late June-early September).

The WSPs provided a primary disinfection treatment of the wastewater with a 2-3 log removal of generic indicator *E. coli*. The bacterial pathogens *Salmonella* spp., pathogenic *E. coli*, *Listeria monocytogenes*, but not *Campylobacter* spp. and *Helicobacter pylori*, were detected in the untreated and treated wastewater, indicating human pathogens were not reliably removed. Seasonal and annual variations in temperature significantly ($p<0.05$) affected the disinfection efficiency. Improved disinfection and pathogen removal was observed for the two-cell system in Clyde River as compared to the one-cell system in Pond Inlet. A quantitative microbial risk assessment should be performed to determine if the release of low levels of human pathogens into the arctic environment poses a human health risk.

Keywords: Wastewater treatment, Arctic Canada, municipal wastewater, disinfection, fecal indicator bacteria, bacterial pathogens, wastewater temperature

INTRODUCTION

In Canada's Arctic regions (Nunavut, Nunavik and Northwest Territories), wastewater stabilization ponds (WSPs) continue to be the most common wastewater treatment solution to manage municipal wastewater. Since there is no need to add chemical flocculants and install mechanical equipment to aerate and mix the wastewater, the WSPs can be easily operated and maintained despite a limited capital and operational budget. However, WSPs, which are completely reliant on un-aided natural biological processes to treat wastewater, may experience treatment limitations due to the harsh arctic climate and not be able to achieve treatment goals set out by Canada-wide strategy for the management of municipal wastewater in the new Wastewater System Effluent Regulation (Environment Canada 2015).

In the majority of Nunavut's 25 small and remote communities, WSPs operate as retention lagoons with no discharge during the winter period. During the nine months of winter the perimeter of the ponds (surface, walls and floor) are frozen while the interior liquid hovers around the freezing point. In June the ponds begin to thaw, and the entire volume remains liquid until freeze-up starts in September. This period of 2-3 months is called the "treatment season", as it characteristically has higher biological activity (phytoplankton and bacteria) due to warmer air temperatures and extended daylight yielding elevated water temperatures. In September before the freeze-up, the contents of the ponds are discharged either directly into the aquatic receiving environment or to a natural tundra wetland for further polishing.

Release of inadequately treated wastewater with a content of human infectious pathogens into the environment may pose a potential human health risk. People living in Nunavut communities may particularly be at risk as their diets are reliant on the local harvest of food from traditional sources (Daley et al. 2014). In addition, recreational activities often take place near

wastewater effluent discharging areas (Harper et al. 2011, Daley et al. 2017). Finally, the overcrowded housing in these communities may be exacerbating the frequency of inter-person spread of infectious agents (Goldfarb et al. 2013, Harper et al. 2011). From a public health perspective, it is important to investigate whether current WSPs in Nunavut achieves compliance with disinfection goals and removal of human bacterial pathogens to minimize the pathways for the transmission of infectious diseases.

Many bacterial pathogens have been associated with waterborne diseases, including the enteric pathogenic *Escherichia coli* serotypes such as O157:H7, *Salmonella* spp., *Campylobacter* spp., *Helicobacter pylori* and the non-enteric, environmental *Listeria monocytogenes*. Outbreaks of enterohemorrhagic *E. coli* (EHEC) O157:H7 have occurred in Canada's northern communities. While the original source of infection was not identified, person-to-person transmission was in both cases suggested as a significant risk factor (Rowe et al. 1994, Orr et al. 1994). Goldfarb et al. (2013) tested 86 stool specimens, which had been obtained from patients with diarrhea at the hospital in Iqaluit (NU), for the presence of 50 different bacterial, viral and parasitic pathogens. They detected *Salmonella* spp. and *Campylobacter* spp. in 47% of the specimens, indicating that outbreaks of *Salmonella* spp. and *Campylobacter* spp. may have occurred. *H. pylori* infections have arisen as an emerging health concern in communities in the Canadian Arctic with the detection of the bacterium in community water supplies in Chesterfield Inlet (NU) and Repulse Bay (Nauyasat, NU) (Lefebvre et al. 2013, Goodman et al. 2008, McKeown et al. 1999). The cold-tolerant *L. monocytogenes* is mainly associated with foodborne outbreaks such as the large outbreak in 2008 in Ontario, Canada (Weatherill et al. 2009). This environmental bacterium can be readily isolated from fresh water in Southern Canada (Stea et al. 2015, Lyautey et al. 2007) while its presence in the arctic environment is unknown.

At the present time, there is a lack of information regarding the removal of fecal indicator bacteria (i.e., disinfection) and human bacterial pathogens in WSPs in Nunavut. To close this knowledge gap, the objectives of the present study were to determine if treatment of municipal wastewater in arctic WSPs successfully removes fecal indicator bacteria (*E. coli*) and selected human bacterial pathogens (pathogenic *E. coli* serotypes (e.g., O157:H7), *Salmonella* spp., *Campylobacter* spp., and *H. pylori*, and the non-enteric *L. monocytogenes*). The treatment efficacy was investigated over three years (2012-2014) in the two remote communities in Nunavut, Pond Inlet and Clyde River, which are serviced by WSP treatment systems consisting of a single cell and two cells, respectively.

MATERIALS AND METHODS

Study sites

From September, 2012 to September, 2014, seven sampling trips were made to Pond Inlet (latitude 72° 41' 57" N, longitude 77° 57' 33" W; population: 1549 [Statistics Canada 2012]) and another six sampling trips to Clyde River (latitude 70° 28' 26" N, longitude 68° 35' 10" W; population: 934 [Statistics Canada 2012]). Both Pond Inlet and Clyde River are remote fly-in communities that are located on the eastern shore of Baffin Island, in Nunavut's Qikiqtani region. Both communities have polar arctic climates with long cold winter and short cool summers. Based on 1981 to 2010 Canadian climate normals, February is the coldest month with daily average temperatures of -34 °C in Pond Inlet and -30 °C in Clyde River, while July is the warmest month with daily average temperatures of 6 °C in Pond Inlet and 5 °C in Clyde River

(Environment Canada 2014). Most of people living in these communities are Inuit, who follow a traditional lifestyle of hunting and fishing.

Pond Inlet employs a one-cell engineered WSP, which was commissioned in 2005 and is located approximately 1.4 km to the east of the hamlet. All wastewater generated is delivered by trucks to the WSP. The treated wastewater is discharged from the WSP once annually, usually over a three week period starting in September and ending in early October just prior to freeze-up. The wastewater effluent exits the WSP over the berm and travels through a ditch and then down a steep hill (500 m) before arriving in the ocean receiving environment (Eclipse Sound). The WSP was designed to be a facultative pond with a surface area of approximately 4 ha and an average depth of approximately 1.9 m during the summer. The estimated volume of wastewater effluent discharged is 8.0×10^7 L. Traditional uses of the ocean receiving environment include fishing and hunting. During the summer season, especially in July and August, schools of Arctic char migrate past the wastewater effluent discharge point. The timing of the annual decant is therefore timed to coincide with the departure of the Arctic char from the area. In addition, during the August and September sampling trips, hunting of narwhals and seals in the nearshore environment surrounding the community was observed.

Clyde River recently expanded their WSP system to include a larger, secondary pond in 2011. The original WSP, the primary pond, had due to the increasing population become too small to accommodate the annual wastewater volume. Therefore, the secondary pond was built to increase the wastewater holding capacity. The expected annual wastewater volume is 3.1×10^7 L using the assumption that each of the 934 inhabitants produces 90 L of wastewater per day. The intended use of the two-cell WSP system is a scheme where the raw wastewater is dumped into the primary pond to enable settling and precipitation processes. At regular intervals, pre-

processed wastewater should then be transferred from the primary pond into the secondary pond to receive further treatment before the annual decant from the secondary pond, where treated wastewater is passed through a vegetated filter strip before going into the ocean receiving environment (Patricia Bay).

Sampling strategy

The same sampling strategy was used in Pond Inlet and Clyde River during visits from September 2012 to September 2014, where representative samples were obtained of raw wastewater from trucks, wastewater in different parts and depths of the WSPs and treated wastewater just prior to the decant (Clyde River) or during the decant (Pond Inlet). In Pond Inlet, outfall samples, i.e., the effluent just prior to entry into the ocean receiving environment, were also collected. In addition, the ocean samples from the immediate vicinity of the outfall point, were collected before and during the decant event in Pond Inlet. Specifically, four outfall samples were collected in both 2013 (two samples) and 2014 (two samples) during the decant event. Six ocean samples from the immediate vicinity of the outfall point, were also collected before (three samples) and during the decant event (three samples) in Pond Inlet in September 2014.

The first trip to Pond Inlet and Clyde River was the end of the summer treatment season in 2012. The WSP in Pond Inlet was sampled at the start, middle, and end of the summer treatment season, including decant events, in 2013 and 2014. Both the primary and secondary ponds in Clyde River were sampled at the start, middle, and end of the summer treatment season in 2013, while in 2014 the ponds were sampled at the start and end of the summer just prior to the decant event. The sampling events representative of the start, middle, and end of the summer treatment

season took place late June/early July, late July/early August, and early/middle September, respectively.

Continuous WSPs monitoring parameters collection

Deployment of multi-parameter sondes (YSI Inc., Yellow Spring, OH) allowed for *in-situ* measurements of wastewater temperature, pH, and dissolved oxygen (DO). During the first sampling trip in each year, the sondes were installed in the WSPs to record the parameters hourly until the sondes were retrieved at the end of the treatment season. In addition, HOBO temperature/light pendants, temperature/water level loggers and ROX DO probes (Onset Computer Corporation, Cape Cod, MA) were installed at various depths of the WSPs to capture parameters and also to validate the continuous recording measured by the *in-situ* sondes.

Degree days calculation

‘Degree days’ is a concept used in the agricultural field to indicate the accumulative effect of temperature on the growth potential of plants in a specific geographical site. Use of degree days also allows for comparison of biological activity in wastewater treatment carried out in different geographical sites with different climates (Ragush et al. 2015). To calculate degree days in order to study how temperature influenced the disinfection performance of WSPs in Pond Inlet and Clyde River, the surface wastewater temperatures were used. The calculation of degree days involves averaging temperature measurements for each day and then subtracting the reference temperature. In this study, the reference temperature was chosen as 5 °C. For example, if the average temperature at a specified day 1 was 10 °C then this would lead to a degree day value for that day of 5 ($10\text{ °C} - 5\text{ °C} = 5\text{ °C}$). In this calculation, only days with average temperature above

5 °C are considered, meaning that on days where, for example, the average temperature is 2 °C, the degree day value would be recorded as 0. To obtain the total degree day values for a certain number of days, the number of degrees for each day is summed up for the specified period, i.e., the degree days for a period of three days would be 6 (5+1+0) if the degree days were recorded as follows on day 1=(10-5 °C)=5, day 2=(6-5 °C)=1, day 3=(2-5 °C)=0.

Microbiological sample analysis

Wastewater samples were obtained in sterile 1 L or 500 mL containers (Nalgene, Fisher Scientific, Nepean, ON, Canada), stored in a cooler (4 °C) and flown to the Northern Water Quality Laboratory (NWQL) at the Nunavut Research Institute in Iqaluit, NU. Upon arrival to NWQL, the analysis for the content of fecal indicator bacteria (*E. coli*) was performed immediately. Samples were also flown to Halifax, NS for the commencement of the selective enrichment for pathogenic bacteria within 24 to 48 hours. DNA was also extracted from wastewater samples within 24 hours of the original sampling event. The immediate processing was done to minimize changes in the microbiology of the samples due to the transportation time.

Enumeration of fecal indicator bacteria (*E. coli*)

Fecal indicator bacteria (*E. coli*) were enumerated using the American Public Health Association (APHA) Standard Method 9223 (American Public Health Association 1998). Samples were processed using Colilert[®]-18 and Quanti-Trays/2000[®] following the manufacturer's procedure (IDEXX Laboratories, Inc., Westbrook, ME, USA). The result was log transformed and expressed as Log MPN/100 mL.

Kinetics of *E. coli* removal

First order rate constants were calculated to estimate *E. coli* removal rates based on the assumption of a completely mixed batch reactor, an assumption which was supported by water quality results from both Pond Inlet and Clyde River WSPs. This first order rate constant was a conservative estimate of *E. coli* removal rates because of the limited sample size. The impact of not including the continuous addition of raw wastewater was expected to be small, because the additional wastewater being added during the treatment period (ranging from 31-34 days in Pond Inlet (start to middle of the treatment season) and 64-74 days in Clyde River (start to end)) only represented 1/12th of the annual wastewater volume in Pond Inlet, and 1/6th of the annual wastewater volume in Clyde River. Therefore, the actual rate constant would be expected to be higher than the conservative rate constant.

The first order rate constant for *E. coli* removal was calculated as follows:

$$K = -\ln(C_t/C_0)/t$$

Where:

K is the first order rate constant (1/day)

C₀ is *E. coli* concentration (Log MPN/100 mL) at the beginning season in the WSP

C_t is *E. coli* concentration (Log MPN/100 mL) at the middle season for Pond Inlet and at the end season for Clyde River WSPs

t is the time interval between the two treatment seasons (days)

Detection of bacterial pathogens presence/absence and concentrations by TaqMan quantitative polymerase chain reaction (qPCR) assays.

Duplicate wastewater samples (10 mL each) were subjected to an initial pathogen enrichment step in Fraser, Bolton, Rappaport-Vassiliadis, buffered peptone water for *L. monocytogenes*, *Campylobacter*, *Salmonella* and pathogenic *Escherichia coli* serotypes, respectively. The enrichment steps were carried out using previously published protocols (Stea et al. 2015a and 2015b). Following enrichments, 2 mL from each of the enrichment broths were combined and added into a 15 mL sterile test tube and were centrifuged at 3200 x g for 10 minutes to obtain cell pellets for DNA extractions. DNA was extracted from cell pellets using the PowerSoil MoBio kit (MoBio, Carlsbad, CA, USA) following the manufacturer's instructions with a final volume of 100 µL. Each qPCR reaction (25 µL) consisted of 7.7 µL of DNase-free water (Fisher Scientific), 12.5 µL of TaqMan master mix (Applied Biosystems Fast Advanced 2X, Applied Biosystems), 0.3 µL each of 10 µM forward and reverse primers, 0.2 µL of 10 µM TaqMan hydrolysis probes, and 4 µL of sample DNA. The qPCR primers, TaqMan hydrolysis probes, running conditions for *Campylobacter* spp., *Salmonella* spp., pathogenic *E. coli* and *L. monocytogenes* were described in Lund et al. (2004), Cheng et al. (2008), Ibekwe et al. (2002), Rodriguez- Lazaro et al. (2004) and Stea et al. (2015b), respectively, and also listed in the supplemental material (Table S1) together with details of the qPCR conditions. Bioinformatic analysis revealed that the Ibekwe et al. (2002) method, which targets the *eae* gene (intimin), detected enterohemorrhagic and enteropathogenic *E. coli* (e.g., O157:H7, O145:H28, O55:H7 and O111:H7, see the full list and *eae* amplicon alignment in the supplemental material, Figure S1). Positive controls contained DNA that were extracted from *Salmonella* Typhimurium (ATCC 14028, Manassas, VA, USA), *E. coli* O157:H7 (strain EC 961019, kindly provided by H. Schraft, Lakehead University, Thunder Bay, ON, Canada), *Campylobacter jejuni*, *C. lari*, and *C. coli* strains (kindly provided by L. Waddington, Canada Food Inspection Agency, Dartmouth, NS,

Canada), and *L. monocytogenes* 568 (serogroup IIa). Negative controls consisted of DNA extracted from sterilized enrichment media. Each qPCR run contained positive, negative, and non-template controls, and samples. The qPCR detection was performed in a StepOne Plus system (Applied Biosystems). The results were reported as the presence/absence of each pathogen in 10 mL of wastewater originally used in the pathogen enrichment protocols.

To quantify pathogen cell numbers in each sample, 100 mL wastewater volumes were centrifuged at 3200 x g for 10 minutes to harvest microbial cells. DNA was extracted from the cell pellets using the PowerMax Soil DNA isolation MoBio kit (MoBio, Carlsbad, CA, USA) following the manufacturer's instructions. The final volume for each DNA extract was 100 μ L. In addition to detection of the pathogens mentioned above, the presence/absence of *H. pylori* was also analyzed following a protocol based on that from He and other researchers (2002) using DNA extracted from *H. pylori* 26695 (ATCC 700392D-5) as the positive control (see Table S1 for details on the method).

Standard curves, which allow for the quantification of each pathogenic bacterium in samples collected from 2013 to 2014 treatment seasons, were created. DNA was extracted from ten-fold dilution series of positive control cultures (10^8 to 10^0 CFU/mL) in tryptic soy broth (TSB, BD-Difco). Prior to the DNA extraction, 10-mL volumes of each dilution of the positive control samples were pelleted at 3200 x g for 10 minutes followed by DNA extraction using the PowerSoil MoBio kit (MoBio, Carlsbad, CA, USA), with a final elution volume of 100 μ L. The TaqMan qPCR assays were performed as described above. The obtained standard curves for all pathogenic bacteria had qPCR efficiencies ranging from 82% to 108%, with R^2 values ranging from 0.986 to 0.998. Two technical replicates were run for all standards, samples, negative controls (DNA extracted from 10 mL of sterile TSB), non-template controls and the difference of

the threshold cycle (Ct) value between the replicates was less than 0.5. The limit of detection (LOD) of the qPCR assay was determined to be 1 CFU/mL for *Salmonella* spp., 1 CFU/mL for *C. jejuni*, *C. lari* and *C. coli*, respectively, 10 CFU/mL for *L. monocytogenes*, and 10³ CFU/mL for pathogenic *E. coli* serotypes. Quantity of approximate cell numbers for each pathogenic bacterium was reported as Log CFU/100 mL. The absence of PCR inhibitors in the DNA extracts was confirmed in experiments with each positive bacterial strain spiked into wastewater samples (data not shown).

Confirmation of the presence of pathogenic *Campylobacter* spp. by triplex polymerase chain reaction (PCR)

The TaqMan assay (Lund et al. 2004) was designed to detect six species of *Campylobacter*. Samples that tested positive for *Campylobacter* spp. in the TaqMan qPCR assay were further analyzed for the presence of *C. jejuni*, *C. lari*, or *C. coli* in a triplex PCR method (Khan and Edge 2007). PCR reactions (25 µL) contained 12.5 µL of master mix (Taq 2X Master Mix, New England Biolabs), 0.5 µL of each 10 µM forward and reverse primers, 1 µL of sample DNA and 8.5 µL of Dnase-free water. The triplex PCR reactions contained the following forward and reverse primers: J-UP/J-DN for detection of *C. jejuni* (349 bp), L-UP/L-DN for detection of *C. lari* (279 bp), and C-UP/C-DN for detection of *C. coli* (72 bp) and was performed in a T-Gradient thermocycler (Biometra). The PCR condition had initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 45.6 °C for 30 seconds, extension at 68 °C for 45 seconds, and had a final extension at 68 °C for 5 minutes. Each PCR run contained positive controls (DNA from *C. jejuni*, *C. lari*, and *C. coli*), samples and non-template controls. PCR products were detected by 2% agarose gel electrophoresis. For

the TaqMan assay to detect *Campylobacter* spp., the detection limit for the enriched sample was 1 CFU/10 mL (enriched to at least 50 CFU/mL of Bolton enrichment broth), while for the triplex PCR, the detection limit was 1 CFU/mL for *C. jejuni*, *C. coli*, *C. lari*, respectively.

Statistical analysis

The normality of the data presented in this paper was checked by D'Agostino-Pearson omnibus normality test in Prism 7 (version 7.0b, Graph Pad Software, Inc., La Jolla, CA, USA). The test result showed that the data did not follow a normal distribution. Differences between two groups were therefore tested with the non-parametric t-test (Mann-Whitney test) while differences among three groups were tested using the non-parametric one-way ANOVA test (Kruskal-Wallis test). The Spearman rank correlation test was used to assess the correlation between the concentrations of *E. coli* and other related wastewater parameters. All the tests mentioned above were performed in Prism 7 (version 7.0b, Graph Pad Software, Inc., La Jolla, CA, USA). Differences among Spearman rank correlation coefficients (r_s) were considered significant if $p < 0.05$.

RESULTS AND DISCUSSIONS

Pond environment and wastewater quality in the one-cell and two-cell arctic WSPs

The pond surface temperature, pH and DO profiles obtained from the Pond Inlet and Clyde River WSPs during the 2012, 2013 and 2014 treatment seasons are presented in Figures 1 and 2, respectively.

In Pond Inlet, the temperatures gradually increased from the beginning to the middle of the treatment season (2012: 13.1 to 16.9 °C; 2014: 11.1 to 17.8 °C) followed by a decrease to 4.3-5.4 °C at the end of the season in 2012 and 2014 (Figure 1a). A similar trend was seen in 2013, except the temperature fluctuated during the last part of the season from 15.9 °C to 8.8 °C, followed by an increase to 14.6 °C and then a gradual decrease to 2.0 °C at the end. In 2012, temporal spikes in pH-values were observed in the WSP where pH rose from 7.5 to 8.1 mid-season (Figure 1b), suggesting algal growth. The pH stayed at about 7.7 for the remainder of the treatment season. In 2013, however, the pH gradually increased from 7.2 to 7.6 over the treatment season with no apparent spikes. In 2014, the pH gradually increased from 7.6 to 8.0 mid-season and followed by a slow decrease to 7.7. Interestingly, the constant low levels of DO close to or below 0.2 mg/L through the entire summer season in 2012 (Figure 1c) contradicted the presence of algal growth that was indicated by pH measurements that year.

In Clyde River, the pond surface temperatures similarly peaked mid-season (Figure 2a). For example, during the sampling trip in 2013, the highest temperature of 13.7°C was observed in mid-July compared to 6.2°C in the late June and 2.9°C in September. Figure 2b shows pond pH-values in 2014 exhibited a small peak going from 7.4 to 7.8 around mid-season after which the pH stabilized at 7.4-7.5 for the remainder of the season. Similarly to observations in Pond Inlet WSPs, pH-values in Clyde River gradually increased from 7.3 to 7.6 during the 2013 treatment season. DO levels consistently remained below the detection limit (0.2 mg/L) in both 2013 and 2014 (data not shown), suggesting that the secondary pond remained anaerobic during the summer treatment seasons for two consecutive years.

An assessment of efficiency of the wastewater treatment offered by the one-cell Pond Inlet and two-cell Clyde River WSPs revealed that the anaerobic ponds effectively removed total

suspended solids (TSS) to approach the Canadian municipal wastewater standards (25 mg/L), however, removal of carbonaceous biochemical oxygen demand (CBOD₅) was limited due to low temperatures and anaerobic environments within the WSPs (Ragush et al. 2015). Taken together, it appeared that the WSPs only delivered limited primary treatment when it comes to the removal of nutrients. While WSPs in both communities were intended to operate as facultative ponds, this was not consistently the case, likely due to the cool arctic summers.

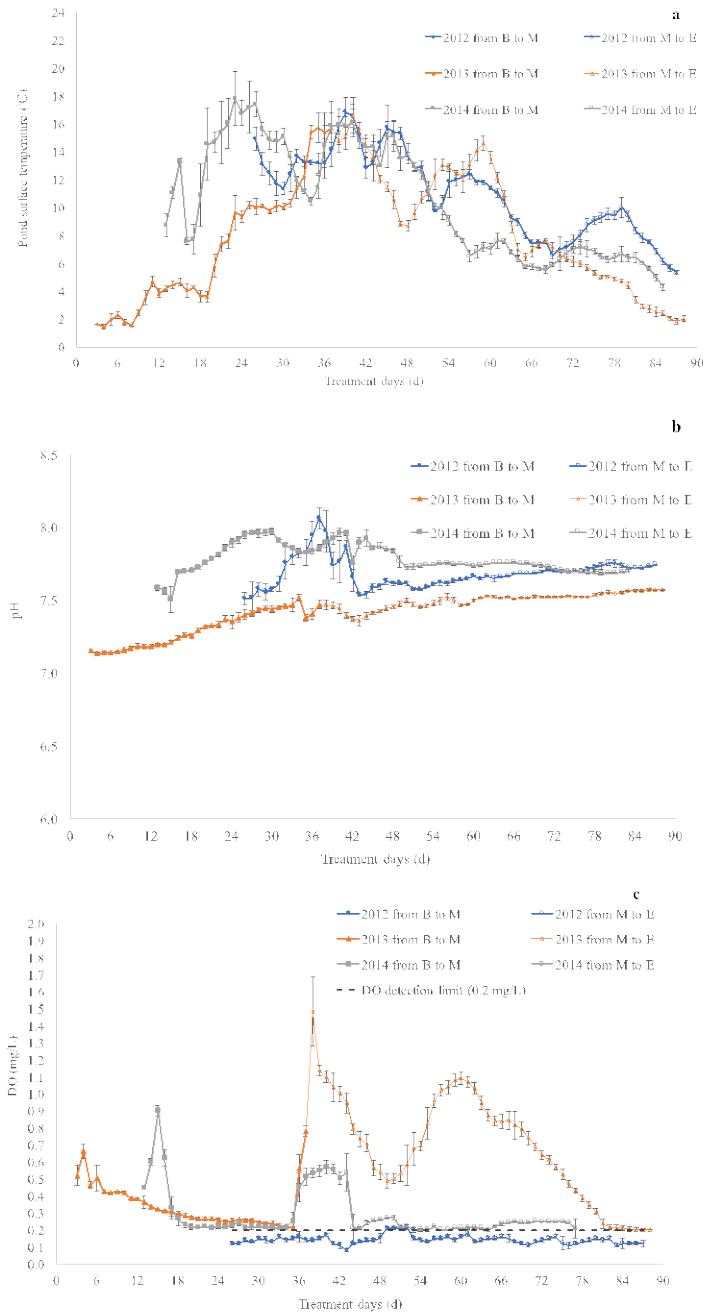
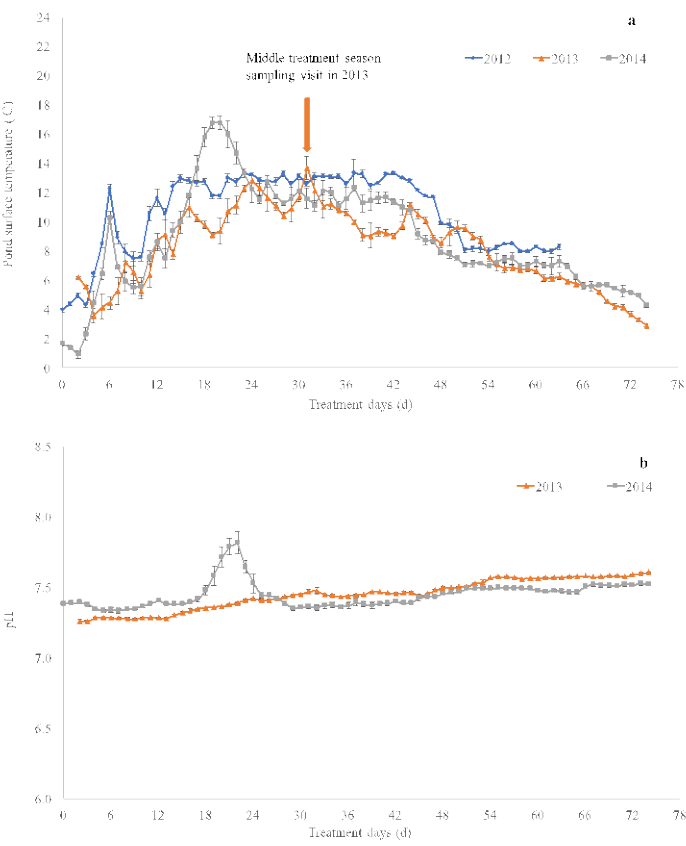


Figure 1. Environment in the Pond Inlet waste stabilization pond during the treatment seasons of 2012, 2013 and 2014 shown by a) the surface pond temperature, b) wastewater pH and c) DO concentrations. Each data point represents daily averages of hourly measurements (n=24, mean \pm standard deviation). Closed symbols indicate values obtained between the beginning (B) and middle (M) of the treatment season while the open symbols indicate values obtained between M and the end (E) of the treatment season.



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Figure 2. Environment in the secondary waste stabilization pond in Clyde River during the treatment seasons of 2013 and 2014 shown by a) the surface pond temperature (also 2012) and b) wastewater pH. Each data point represents daily averages of hourly measurements (n=24, mean \pm standard deviation). The DO levels consistently remained below the detection limit (0.2 mg/L) and the data is therefore not shown.

Disinfection treatment in arctic WSPs

Removal of *E. coli*

In Pond Inlet, *E. coli* levels were on average reduced by 1.5 Log MPN/100 mL as raw wastewater levels of 7.2-7.5 Log MPN/100 mL were reduced to final *E. coli* levels averaging 5.8

Log MPN/100 mL in the effluent (Figure 3), which was within the permitted 4-6 Log MPN/100 mL in the current territorial effluent standards (Nunavut Water Board 2014). In the early to mid-season of 2012 and 2013, CBOD₅ and TSS levels exhibited a strong relationship with the reduction of *E. coli* concentrations to the lowest levels of 5.3 Log MPN/100 mL, as indicated by the Spearman Rank Correlation coefficients (r_s) of 0.64 and 0.75 ($p < 0.05$), respectively. However, in the later part of the treatment season, i.e., from late July/early August to early/middle September, *E. coli* levels rose significantly ($p < 0.05$) from 5.3 to 5.9 Log MPN/100 mL. Taken together, it appeared that the disinfection (i.e., *E. coli* removal) and removal of suspended solids (TSS) and nutrients (CBOD₅) (Ragush et al. 2015) were optimal in the middle of the treatment season.

In Clyde River, just prior to decant in September, *E. coli* similarly reached levels in the secondary pond that met the current territorial effluent standard (Nunavut Water Board 2014). *E. coli* concentrations in the raw wastewater in Clyde River ranged from 6.7 to 7.3 Log MPN/100 mL with no significant differences ($p > 0.05$) among sampling events (Figure 4). Treatment in the primary pond removed an average 1.1 Log MPN/100 mL from the raw wastewater resulting in average *E. coli* concentrations of 5.9 Log MPN/100 mL in wastewater samples from the primary pond. Within the secondary pond, there was a significant ($p < 0.05$) reduction of 1.5 Log MPN/100 mL seen from initial levels in June of 5.5 Log MPN/100 mL to 4.0 Log MPN/100 mL in September, yielding an overall 3 log reduction in the *E. coli* concentration during the 2012-2014 treatment seasons. Reductions in TSS levels correlated ($p < 0.05$) with the reduction of *E. coli* concentrations in the secondary pond as indicated by the Spearman Rank Correlation coefficient values (r_s) of 0.75 and 0.74 in 2012 and 2014, respectively. In 2013, there was a weak correlation relationship ($r_s = 0.45$) between the reduction of TSS and *E. coli* levels in the

secondary pond, which may be due to the direct discharge of raw wastewater into this pond
observed during sampling trips in 2013.

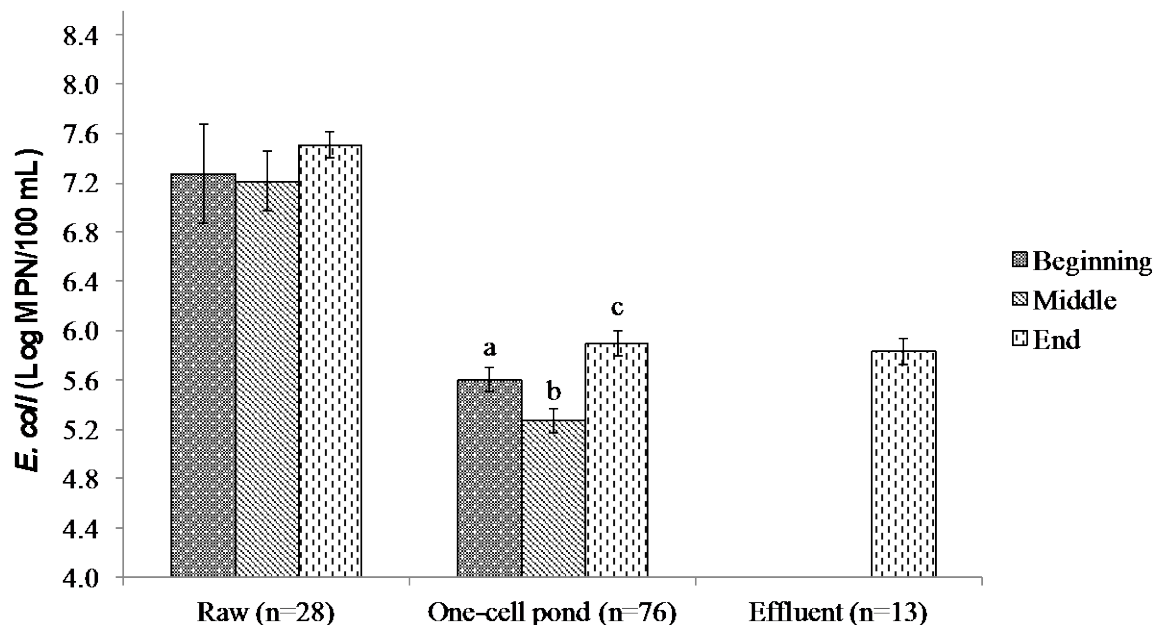


Figure 3. The average *E. coli* levels (Log MPN/100 mL) measured in raw (untreated), one-cell pond and effluent wastewater samples in Pond Inlet in the beginning, middle and end of the 2012 to 2014 treatment seasons. Error bars indicate the standard deviation. Different letters within the same sampling site indicate significant differences ($p < 0.05$) as determined by the Kruskal-Wallis test.

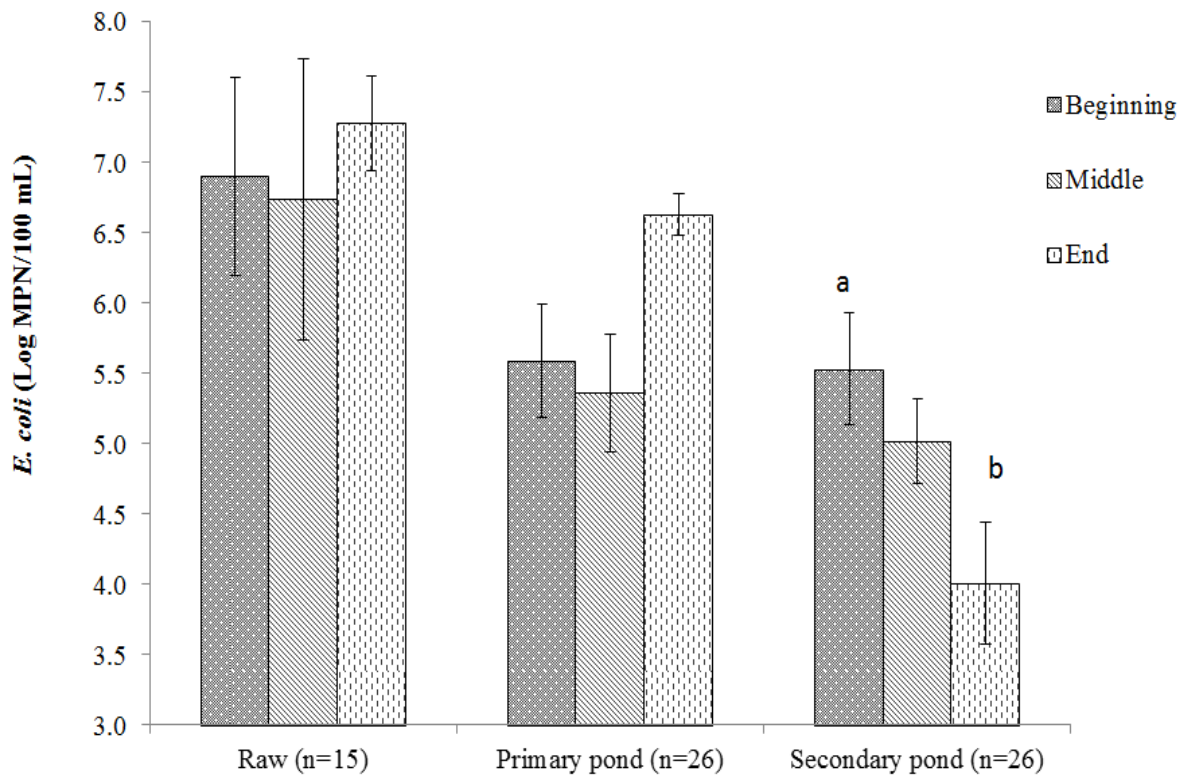


Figure 4. The average *E. coli* levels (Log MPN/100 mL) in raw (untreated) wastewater and samples from the primary and secondary ponds in Clyde River obtained during the 2012 to 2014 treatment seasons. Error bars indicate standard deviations. Different letters within the same sampling site indicate significant differences ($p<0.05$) as determined by the Mann-Whitney test.

Kinetics of *E. coli* removal

The kinetics of the removal of *E. coli* over the different treatment seasons was compared by calculating the first order rate constants in the two WSP systems (Table 1). It should be noted that the first order rate constants were only computed from time periods where the levels of *E. coli* were decreasing.

Table 1. The first order rate constants (k) for *E. coli* removal in Nunavut WSPs

Location	Year	K (1/day)	Duration of summer treatment (days)	Degree days above 5 °C
Pond Inlet	2012	$1.4 \times 10^{-4A} \pm 2.5 \times 10^{-6a}$	33	251
	2013	$1.7 \times 10^{-3B} \pm 5.1 \times 10^{-5}$	34	280
	2014	$3.3 \times 10^{-3C} \pm 1.7 \times 10^{-5}$	31	308
Clyde River (Secondary pond)	2012	$5.6 \times 10^{-3D} \pm 8.1 \times 10^{-5b}$	64	324
	2013	$3.7 \times 10^{-3E} \pm 1.6 \times 10^{-5}$	72	246
	2014	$4.6 \times 10^{-3F} \pm 3.3 \times 10^{-5}$	74	300

A-F: different letters in the same column indicated that the significant differences ($p < 0.05$) were detected by the Kruskal-Wallis test.

^a: average of calculated k values in Pond Inlet between two sampling events from 14 biological replicates with two technical duplicates (mean \pm standard deviation)

^b: average of calculated k values in Clyde River between two sampling event from eight biological replicates with two technical duplicates (mean \pm standard deviation)

Pond Inlet exhibited significantly different ($p < 0.05$) first order rate constants for *E. coli* removal from the beginning to the middle of the treatment season in each of the study years (Table 1) with the highest first order rate constant occurring in 2014, followed by 2013 and then 2012. Since previous studies found that temperature plays an important role in inactivation of *E. coli* in WSPs (Curtis et al. 1992, Davies-Colley et al. 2000, Klock et al. 1971, Marais et al. 1974), the degree days above 5 °C were calculated for these time periods. The trend of degree days above 5 °C indicated that the pond in 2014 (308 degree days above 5 °C) experienced a relatively warmer environment than in 2013 (280 degree days above 5 °C) and 2012 (251 degree days above 5 °C) and offers a possible explanation for observed differences in the first order rate constants for *E. coli* removal over the three study years. This finding agreed with past WSP studies in non-arctic regions, which also showed the importance of temperature in *E. coli* die-off kinetics (Marais et al. 1974, Polprasert et al. 1983, Klock et al. 1971). The seasonal and annual

variations in pH and DO (Figures 1b and 1c) also appeared to relate to disinfectant treatment efficiencies, for example, in 2014 a drop in *E. coli* levels coincided with increased pH (7.6 to 8.0) and DO (0.2 to 0.6 mg/L) levels. Previous studies have shown that pH values exceeding 9, and increased DO levels, effectively removed fecal coliforms including *E. coli* in WSPs (Curtis et al. 1992, Parhad and Rao 1974, Pearson et al. 1987).

In Clyde River, the first order rate constant for *E. coli* removal was highest in 2012 and lowest in 2013, which again appeared linked to a comparatively warmer environment in 2012 compared to the other years (Table 1). For this community, however, pH levels were relatively stable and DO levels were constantly below the detection limits, indicating that algae were unlikely to grow (Figure 2). Overall, the observed differences in *E. coli* removal kinetics indicated annual variations in disinfection treatment performance within the same passive treatment system and geographical location, which may in part be due to local climatic fluctuations.

Removal of human bacterial pathogens in arctic WSPs

WSP temperature and removal of pathogens

The presence of human bacterial pathogens in the Pond Inlet WSP during the 2014 treatment season is depicted in Figure 5. It should be noted that a similar trend was seen in 2013. The non-enteric environmental pathogen *L. monocytogenes* was consistently present in 100% of the samples throughout the treatment season. Results showed that in late June, the enteric pathogens *Salmonella* spp. and pathogenic *E. coli* serotypes were present in 88% and 100% of the samples, respectively. However, mid-season only 55% of samples contained *Salmonella* spp. while 72%

of samples tested positive for pathogenic *E. coli* serotypes. On the last visit in conjunction with the annual decant, these numbers rose back up to 79% and 100% of the samples testing positive for *Salmonella* spp. and pathogenic *E. coli* serotypes, respectively. The other pathogens, *C. jejuni*, *C. lari*, *C. coli*, and *H. pylori*, were not detected in any samples, indicating that their levels remained below the detection limit.

The seasonal temperature variation had no impact on the presence of *L. monocytogenes*, which is a cold-adapted environmental bacterium previously associated with soil, water, and wastewater (Linke et al. 2014). A study of sludge from Swedish sewage treatment plants similarly showed that *L. monocytogenes* persisted in raw sludge samples (Sahlström et al. 2004). Improved removal of *Salmonella* spp. and pathogenic *E. coli* serotypes was observed mid-season coinciding with the highest environmental temperatures. Therefore, similar to the findings for the fecal indicator *E. coli* removal kinetics, it appeared that the higher WSP temperature measured mid-season in late July/early August (average 13.5 °C) improved the removal of *Salmonella* spp. and pathogenic *E. coli* serotypes. Taken together, this indicates the importance of temperature (degree days) measurements to gauge the disinfection efficiency (i.e., removal of fecal indicator bacteria) and removal of selected human pathogens in the arctic WSPs.

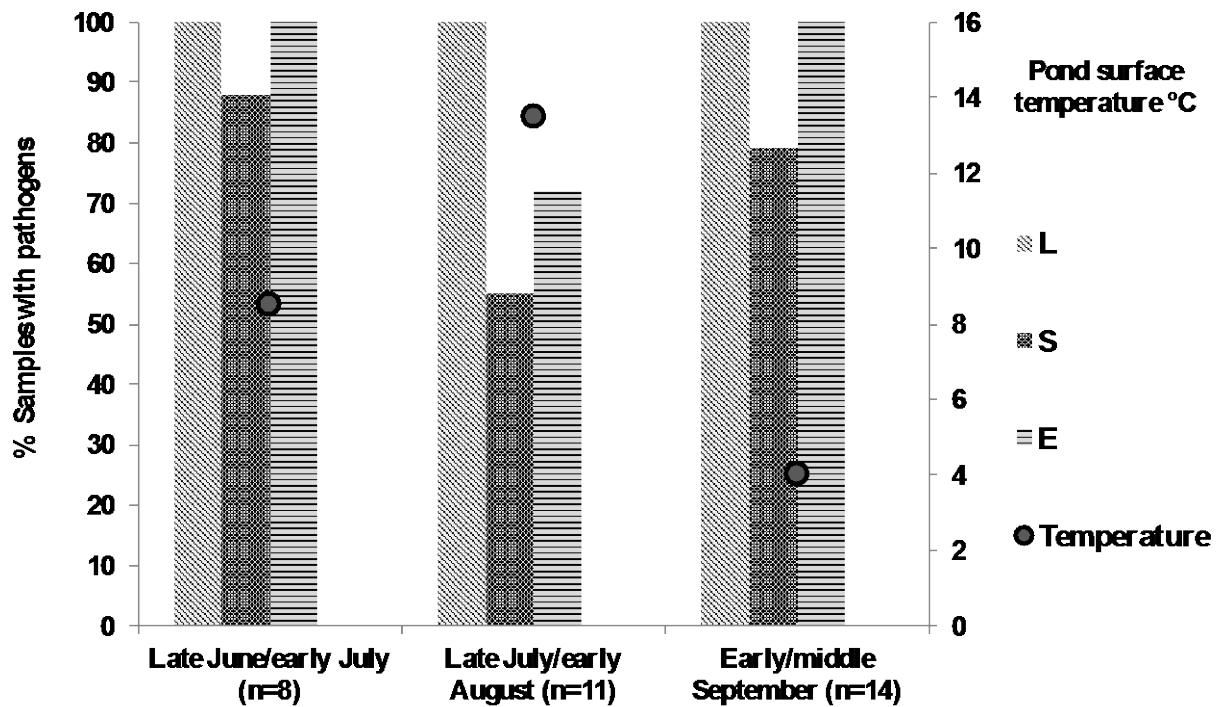


Figure 5. WSP surface temperature and percentage of WSP samples testing positive for the presence of human pathogens during the 2014 treatment season in Pond Inlet. Legend: L - *L. monocytogenes*, S - *Salmonella* spp., and E – pathogenic *E. coli* serotypes.

Removal of human bacterial pathogens along the arctic WSP treatment train

The percentage of the samples testing positive for the presence of as well as the direct counts of human pathogens in raw and treated wastewater samples from the WSP systems in Pond Inlet and Clyde River are shown in Tables 2 and 3, respectively.

Table 2. Percentage of samples testing positive and quantity of human pathogens in raw and treated wastewater samples from the 2013 and 2014 treatment seasons in Pond Inlet.

Year	Wastewater samples (no. enriched samples)	Positive samples (%) following enrichment			Log CFU/100 mL following direct detection (no. positives/total sample no.)		
		L ^a	S ^b	E ^c	L	S	E
2013	Raw (8)	100	88	88	3.8 ^{Ad} ±0.3 (2/4)	4.1 ^A ±0.2 (1/4)	5.5 ^A ±0.5 (1/4)
	WSP (23)	100	78	87	3.4 ^A ±0.2 (7/7)	3.6 ^B ±0.1 (3/7)	4.8 ^B ±0.2 (3/7)
	Effluent (6)	100	83	83	3.5 ^A ±0.3 (4/4)	3.4 ^B ±0.3 (2/4)	4.6 ^B ±0.3 (2/4)
2014	Raw (9)	100	89	100	4.2 ^A ±0.4 (4/4)	4.5 ^A ±0.3 (2/4)	5.5 ^A ±0.4 (3/4)
	WSP (27)	100	74	89	3.5 ^A ±0.5 (7/7)	3.7 ^B ±0.1 (4/7)	4.5 ^B ±0.3 (5/7)
	Effluent (4)	100	75	75	3.6 ^A ±0.4 (4/4)	3.6 ^B ±0.2 (3/4)	4.5 ^B ±0.2 (3/4)

^aL: *L. monocytogenes*

^bS: *Salmonella* spp.

^cE: Pathogenic *E. coli* serotypes

^dDifferent capital letters in the same column indicate that there were significant differences (p<0.05) detected by the Kruskal-Wallis test (mean ± standard deviation).

In Pond Inlet, all three pathogens were detected at levels ranging from 1,000-10,000 copies/100 mL in the September decant (effluent) samples (Table 2). *L. monocytogenes* was consistently present in all raw (untreated), WSP (treated), and effluent samples at unchanged levels, suggesting that this bacterium was not removed in the Pond Inlet WSP. *Salmonella* spp. were present in 88-89% of raw and 74-78% of treated samples in 2013 and 2014, indicating a consistent presence. The concentration of *Salmonella* spp. fell significantly (p<0.05) by 0.5-0.8 Log CFU/100 mL from raw to treated/effluent wastewater samples, indicating some removal in the WSP. Depending on the year, 88 to 100% of raw wastewater samples tested positive for pathogenic *E. coli* serotypes. While the level of positive samples stayed high in the treated

samples, the quantitative analysis revealed that the pathogenic *E. coli* population was reduced by 0.7-1.0 Log CFU/100 mL. The three major *Campylobacter* pathogens (*C. jejuni*, *C. lari*, and *C. coli*) were not detected in neither the enriched samples nor by direct enumeration, indicating a low prevalence in the Pond Inlet wastewater treatment system.

Table 3. Percentage of samples testing positive and quantity of human pathogens in raw and treated wastewater samples obtained in Clyde River in September of 2013 and 2014.

Year (September)	Wastewater samples (no. samples)	Positive samples (%) following enrichment			Log CFU/100 mL following direct detection (no. positive samples)		
		L ^a	S ^b	E ^c	L	S	E
2013	Raw (4)	100	100	100	4.6 ^{Ad} ±0.3 (4)	5.1 ^A ±0.2 (3)	5.2 ^A ±0.5 (3)
	Primary (4)	100	100	100	4.4 ^A ±0.2 (4)	4.9 ^A ±0.1 (3)	4.9 ^A ±0.2 (3)
	Secondary (4)	100	100	100	4.5 ^A ±0.3 (4)	4.6 ^A ±0.3 (3)	4.7 ^A ±0.3 (3)
2014	Raw (4)	100	75	100	4.5 ^A ±0.3 (4)	4.8 ^A ±0.3 (3)	4.9 ^A ±0.4 (3)
	Primary (4)	100	100	100	4.2 ^A ±0.2 (4)	4.4 ^B ±0.1 (3)	4.3 ^B ±0.3 (3)
	Secondary (4)	75	50	75	3.5 ^B ±0.3 (4)	3.6 ^C ±0.2 (2)	3.4 ^C ±0.2 (2)

^aL: *L. monocytogenes*

^bS: *Salmonella* spp.

^cE: Pathogenic *E. coli* serotypes

^dDifferent capital letters in the same column indicate that there were significant differences (p<0.05) detected by the Kruskal-Wallis test (mean ± standard deviation).

In Clyde River in 2013 all three pathogens were detected in all raw sewage and grab samples from both the primary and secondary pond during the September sampling visit, shortly before the annual decant event (Table 3). In 2013, the levels of all three pathogens remained unchanged (p>0.05) along the treatment system. To improve the performance of the two-cell

WSP system in Clyde River, it was proposed based on the treatment suggestions by Dawson and Grainge (1969) and Heinke et al. (1991), that the community use the system in a manner where the smaller primary pond is utilized as a primary treatment cell followed by the transfer of pre-settled wastewater from the primary pond to the secondary, larger pond. Clyde River followed this suggestion in 2012, but returned to dumping raw wastewater into the secondary pond from mid-August to early September in 2013 due to the lack of holding capacity in the primary pond. It may be that this caused the poor disinfection and removal of pathogen performance in 2013; however, 2013 was also a year characterized by lower temperatures (Table 1).

In 2014, Clyde River was able to operate the system according to the recommendations, which led to a reduction of pathogens in treated wastewater samples from the secondary pond. In absolute numbers, this resulted in reductions of one log for *L. monocytogenes*, 0.8 log for *Salmonella* spp., and 0.9 log for pathogenic *E. coli* serotypes. In line with past observations of a relationship between TSS and pathogen removal (Bitton 2011), the current observation of pathogen removal coincided with a significant reduction of TSS observed in the secondary pond (Ragush et al. 2015).

Campylobacter spp. and *H. pylori* was not detected in any of samples during sampling events in Pond Inlet and Clyde River. While their presence might have been expected in the raw sewage (Goldfarb et al. 2013), one possible reason for the absence of *Campylobacter* spp. in the WSP samples is their thermophilic nature, making them vulnerable to the cold arctic climate. It has previously been reported that viable and culturable *Campylobacter* spp. numbers quickly decreased following the discharged of untreated sewage into coastal waters (Jones et al. 1999a). The same study also found that *Campylobacter* spp. suspended in the effluent became unculturable after only 15 minutes of exposure to direct sunlight. During the study period, a high

level of incident solar radiation was measured during sunny days with clear sky in Pond Inlet (Ragush et al. 2015), which may have aided in the inactivation of *Campylobacter* spp.

In terms of the detection of bacterial pathogens in the adjacent environment during the September decant in Pond Inlet, all four outfall samples contained *L. monocytogenes* (average 3.2 Log CFU/100 mL), *Salmonella* spp. (average 2.2 Log CFU/100 mL) and pathogenic *E. coli* serotypes (average 4.1 Log CFU/100 mL). Prior to the decant event, all ocean samples tested negative for the pathogens. However, during the decant event, two pathogens (*L. monocytogenes* and *Salmonella* spp.) were detected in all three ocean samples at average levels of 2.1 and 1.5 Log CFU/100 mL, respectively.

The presence of pathogens in the effluent may pose a risk to human health through various exposure pathways but this will obviously depend on the number and survival of the pathogens being released into the arctic environment and the human and wild-life interactions with impacted areas (Harper et al. 2011, Daley et al. 2017). The predicted infectious dose for pathogenic enterohemorrhagic *E. coli* serotypes such as O157:H7 is only 10 to 100 bacteria (Theron and Cloete 2002), while for *L. monocytogenes* it is 10^7 - 10^8 CFU in healthy hosts and 10^5 - 10^7 CFU in susceptible individuals (Farber et al. 1996). The infectious dose is 10^3 - 10^5 CFU for non-typhoidal *Salmonella* spp. (Bronze and Greenfield 2005, Ryan and Ray 2004). The prevalence of acute gastrointestinal illness (AGI) is reported to be higher in Arctic Canada compared to other parts of the country (Harper et al. 2015), however, the cause of this remains uncertain. Goldfarb et al. (2013) investigated the presence of a range of bacterial, parasitic, and viral agents in patients with diarrhea in Nunavut and commented on their inability to track the source of the observed infectious agents. The source of food and waterborne infectious agents can be local, as in present in locally harvested foods (mammals and fish) or drinking water, or

imported. The latter appeared to have been the case in the *E. coli* O157:H7 outbreak in Arviat (NU) where imported frozen hamburger patties was a likely source (Orr et al. 1994). Few studies exist on the prevalence of pathogens in local food sources. Gauthier et al. (2010) reported that 129 samples of arctic mammals, fowl, fish and community freezers in Nunavik tested negative for the presence of *E. coli* O157:H7 and *Salmonella* spp. While it must be assumed that agents of AGI end up in the municipal wastewater treatment systems, little is known about the potential attributions to human disease and wild-life carriage of pathogens being released with (un)treated wastewater in the arctic. A survey of the release of pathogens into the Antarctic Ocean due to wastewater disposal from Antarctic research stations found that microorganisms released from wastewater remained viable for prolonged periods and thus available for transmission to the local fauna (Gröndahl et al. 2009). Earlier studies had reported the presence of human pathogens (*Salmonella* Enteritidis, *Salmonella* Typhimurium, *Campylobacter jejuni*, and *Pasteurella multocida*) in antarctic seal and bird populations leading the researchers to speculate that the presence of these pathogens could presumptively be attributed to human activity (Broman et al. 2000, Palmgren et al. 2000). Clearly, future studies are needed to uncover whether the release of human pathogens from the discharge of untreated and treated wastewater from arctic communities constitute a human health hazard.

CONCLUSIONS

The study investigated the disinfection and removal of human pathogens in arctic WSPs treating municipal wastewater in Pond Inlet and Clyde River, Nunavut, Canada. The results revealed that WSPs in both communities reduced the content of *E. coli* to levels that are in compliance with the Nunavut Water Board (2014) regulatory limits. The seasonal pond

temperatures appeared to influence the treatment efficiency. The single-cell WSP in Pond Inlet was able to significantly remove *Salmonella* spp. (0.7-0.9 log) and pathogenic *E. coli* serotypes (~1.0 log) but not *L. monocytogenes* from raw to effluent wastewater. The two-cell Clyde River WSP provided better treatment in regards to disinfection and removal of bacterial pathogens with reductions of 1.0-1.5 log, provided the primary pond was used as the only recipient of raw wastewater which then after a settling period was transferred to the secondary pond for further treatment. The best removal of fecal indicator bacteria and pathogens was achieved mid-season in Pond Inlet, likely due to the warmer water temperatures however, due to the traditional and important harvest of seafood at that time of year, the treated wastewater was not released until just prior to freeze-up in September. In spite of the WSP treatment, it should be noted that the bacterial pathogens were still present in levels of 2-4 Log CFU/100 mL in the treated wastewater being discharged into the receiving environment. In summary, arctic WSPs achieved a modest removal of fecal and pathogenic bacteria from municipal raw sewage with some local, seasonal and year-to-year variations. From a public health perspective, it may be prudent to assess the potential risks that the wastewater effluents pose to human health.

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SUPPLEMENTAL MATERIAL

Table S1. Primers and qPCR protocols for detection of bacterial pathogens.

Assay type	Pathogenic bacteria	Primers (F and R) and probes (P)	Sequence 5' to 3'	qPCR protocols
TaqMan	<i>Listeria monocytogenes</i>	HlyQF	CATGGCACCACCAGCATCT	95 °C for 10 min; 40 cycles of 95 °C for 20 sec, 56 °C for 30 sec, 72 °C for 1 min
		HlyQR	ATCCGCGTGTTTCTTTTCGA	
		HlyQP	FAM-CGCCTGCAAGTCCTAAGACGCCA-TAMRA ^a	
	<i>Escherichia coli</i> (pathogenic EHEC/EPEC, intimin+)	EaeF	GTAAGTTACACTATAAAAGCACCGTCG	95 °C for 6 min; 40 cycles of 95 °C for 20 sec, 55 °C for 30 sec, 72 °C for 40 sec
		EaeR	TCTGTGTGGATGGTAATAAATTTTGT	
		EaeP	FAM-AAATGGACATAGCATCAGCATAATAGGCTTGCT-BHQ1 ^b	
	<i>Campylobacter</i> spp.	CampF2	CACGTGCTACAATGGCATAT	95 °C for 6 min; 40 cycles of 95 °C for 15 sec, 60 °C for 1 min
		CampR2	GGCTTCATGCTCTCGAGTT	
		CampP2	FAM-CAGAGAACAATCCGAAGTGGGACA-BHQ1	
	<i>Salmonella enterica</i>	InvAF	AACGTGTTTCCGTGCGTAAT	95 °C for 6 min; 40 cycles of 95 °C for 15 sec, 60 °C for 1 min
		InvAR	TCCATCAAATTAGCGGAGGC	
		InvAP	FAM-TGGAAGCGCTCGCATTGTGG-BHQ1	
SybrGreen	<i>Helicobacter pylori</i>	HPF	TTATCGGTAAAGACACCAGAAA	95 °C for 10 min; 40 cycles of 94 °C for 20 sec, 54 °C for 5 sec, 72 °C for 10 sec
		HPR	ATCACAGCGCATGTCTTC	

a- FAM – fluorescein

b- BHQ1- Black hole quencher



Figure S1. Alignment of the *eae* gene fragment amplified by the Ibekwe et al. (2002) primer pair from different pathogenic *E. coli* serotypes showing the binding site for the EaeP probe (CLC Genomics Workbench Version 9). Shown are enterohemorrhagic *E. coli* (EHEC) strains O157:H7 and O145:H28 and enteropathogenic *E. coli* (EPEC) strains O55:H7 and O111:H7. A search in the NCBI database (<https://www.ncbi.nlm.nih.gov/>) revealed that other EHEC and EPEC serotypes were also detected by this primer and probe set including EHEC strains O79:H7, O91:H14, O153:H2, O156:H25, and EPEC strains O55:K59 , O126:K71,O86:K61, O44:K74, O26:K60, O128:K67, O127:K63, O126:K71, O125:K70, O119:K69, O111:K59, O111:K58, O82:H11, O40:H19, O170:H49, O2:H40, O119:H25, O76:H7, O136:H21.